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Ganoderma lucidum: A Novel Study for Inhibiting Protease Virulence Weapon of Multiple Drug Resistant and Extended Spectrum Beta-Lactamase *Pseudomonas aeruginosa* Clinical Isolate

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ABSTRACT:

Protease enzyme was considered to be one of the most virulence factors produced by multidrug resistant and extended spectrum β-lactamase *Pseudomonas aeruginosa* clinical isolate. Therefore, protease enzyme was isolated followed by purification using ammonium sulphate precipitation and DEAE-Sephadex A-50 chromatography. In purification, 70% ammonium sulphate fractionation gave maximum protease activity 39 u/ml and maximum protease specific activity of DEAE-Sephadex A-50 chromatography purified sample was found to be 84.6 u/mg with purification fold of 9.4. The molecular weight of the enzyme was estimated to be approximately 40 KDa as shown by SDS electrophoretic analysis. Methanol extract of *Ganoderma Lucidum* fruiting bodies has been tested against *P. aeruginosa* showing high antibacterial activity. Then its effect on purified protease enzyme indicated that it is a reversible non-competitive protease inhibitor (kis = 0.45 mg/ml) with high promising activity. The positive results of screening the antibacterial activity of *G. lucidum* extract by inhibition of protease forms a primary platform for further phytochemical studies and development of new drugs for therapy of skin burn infections.

KEYWORDS: *Ganoderma lucidum*; *Pseudomonas aeruginosa*; multidrug resistance; virulence factor; Protease.

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INTRODUCTION:

Gram-negative bacteria are generally resistant to antibiotics and are paramount pathogens. Recently multi-drug resistant (MDR) Gram-negative bacteria have become more prevalent and are causing great problems in treatment of infections [1]. *Pseudomonas aeruginosa* is a Gram-negative rod bacterium, which is reported to be ubiquitous in the natural environment, humans, and animals. In addition, it is one of an important opportunistic human pathogens that causes severe infections in immunocompromised patients [2]. One of the clinical significance of *P. aeruginosa* is its ability to secret several virulence factors. The virulence factors include mucoid exo-polysaccharide, lipopolysaccharide, biofilm, pili, exotoxin A, pigments, lipase, protease, haemolysin, histamine, exoenzyme S, leukocidin and rhamnolipids [3]. These help the bacteria to adhere and invade to their host by damaging the host's immune responses and forming a barrier to antibiotics. Protease enzyme is one of the most important virulence factors secreted by *P. aeruginosa* [4]. *Pseudomonas aeruginosa* has an arsenal of impressively efficient proteases that helps establishing and maintaining an infection [5]. Proteases are crucial virulence factors of *P. aeruginosa*. These proteases work together leading to significant damage of host tissues. The elastolytic activity of these enzymes plays a major role in *P. aeruginosa* pathogenesis because a number of organs or tissues are composed of elastin (e.g. lung tissue, vascular tissue and ocular tissue) and need elastic properties for their physiologic functions. Any treatment targeting these proteases could be a potential therapeutic option [6-8].

Ganoderma lucidum, a basidiomycetes, belonging to the family of *Polyporaceae*, is one of the most famous traditional Chinese medicinal herb. *G. lucidum* (Lingzhi) is a popular medicinal mushroom that has many biologically active components like phenolics, flavonoids, polysaccharides, etc., giving it its antimicrobial, antioxidant, antiviral and anticancer properties [9], cardiovascular, respiratory, antihepatotoxic and antinociceptive effect [10,11].

Not much literature is available with regard to the antimicrobial activities of the fruiting body of *G. lucidum* for treatment of multidrug resistant *P. aeruginosa* producing protease enzyme virulence factor. So, the present study aims to evaluate the efficiency of *Ganoderma lucidum* extract as a novel alternative promising method for attenuation of protease enzyme virulence factor of multidrug resistant *P. aeruginosa* isolated from skin burn infection.

MATERIAL AND METHODS:

A- *Pseudomonas aeruginosa* isolate:

Extended Spectrum Beta-Lactamse Multidrug Resistant *P. aeruginosa* (ESβLMDRPA) clinical isolate of skin burn infection, previously recovered and identified by Khalil *et al*. [12], was tested for its ability to produce protease enzyme as a virulence factor.

The ESβLMDRPA isolate was sub-cultured on cetrimide agar plate supplemented with 15µg/ml naldixic acid, *Pseudomonas* isolation agar for fluorescence (PIAF) and *Pseudomonas* isolation agar for pyocyanin (PIAP) for 18 h at 37°C. Stock culture was stored in 0.05 M K-Na-phosphate buffer, pH 7.0, containing 15% glycerol at –20 °C.

B- Detection of protease activity:

This method was described by Vermelho *et al*. [13] where *P. aeruginosa* isolate was inoculated on medium contains 2% (w/v) sucrose, 0.5% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) KCl and 1.5% (w/v) agar, supplemented and autoclaved with 1% (w/v) bovine serum albumin (BSA). Medium was adjusted to pH 7.0. Twenty ml of culture media was then poured per plate and allowed to harden. 0.1 ml of distilled H_2O was placed in the center of the agar plates, and 0.1 ml of bacterial suspension was streaked upon the plate surface, where 0.1 ml of H_2O was streaked on control plate . After inoculation, the plates were incubated at 37°C and observed daily for ten days. Extracellular protease detection was done after staining with coomassie blue (0.25%, w/v) in methanol-acetic acid-water 5:1:4 (v/v/v), Then destaining required by the use of methanol and acetic acid. Regions of enzyme activity were detected as clear areas (no blue colour), indicating that hydrolysis of the substrates had occurred.

C- Production of protease from *P. aeruginosa:*

Production of protease from *P. aeruginosa* was carried out in a liquid medium containing (g/L) peptone, 5; beef extract, 5; yeast extract, 1; Nacl, 15; agar, 20 and casein, 10. The intial pH of the medium was adjusted to 7, then divided into 100 ml fractions in flasks (250) and autoclaved. Five millitres of suspension was inoculated into 250 ml Ehrlenmeyer flasks containing 100ml sterilized liquid medium and incubated at 35 °C for a period extended to 10 days. Then centrifuged at 6,000 rpm for 20 min after which the supernatant was used as a crude enzyme preparation [14, 15].

D- Protease assay:

Proteolytic activity was determined using 0.6 % Hammersten casein solution (50 mMTris–HCl, pH 7) as a substrate. The enzyme solution (1 ml) was mixed with the substrate solution (5 ml) and incubated at 30 °C for 30 min. The reaction was stopped by the addition of 0.5 ml 20 % TCA and kept for 10 min at 25°C . Then centrifuged at 6.000 rpm at 4°C for 15 min and the absorbance measured at 280 nm. One unit of protease activity is defined as the amount of enzyme required to liberate 1 µg tyrosine per ml per min. The experiments were carried out in triplicates and the mean value expressed as unit protease activity [16].

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E-Purification of Enzyme

i-Ammonium Sulphate Precipitation:

P. aeruginosa was grown for 3 days as described previously. The cells were separated by centrifugation (6000 rpm, 10 minutes), and the supernatant was fractionated by precipitation with ammonium sulfate between 50% and 70% of saturation. All subsequent steps were carried out at 4 °C. All precipitates were collected and dialyzed against 50 mM Tris-HCl buffer pH = 7 for 24 hours using dialysis bag. The dialyzed enzymatic fractions were subjected to protein and protease activity determination. Then the precipitate of the highest ammonium sulphate fraction was applied to gel fitration chromatography [17].

ii-Gel filteration chromatography on DEAE-Sephadex A-50:

The dialyzed enzyme preparation was applied on a DEAE-Sephadex A-50 column (with dimensions 1.5 cm diameter and 20 cm length). The column was pre-equilibrated with 50 mM Tris-HCl (pH 8.0). The sample was eluted with the same buffer. Ten fractions were collected at a flow rate 1 ml/min constant intervals. The most active fractions (6-8) were dialyzed against 50 mM Tris-HCl pH=7 at 4 $^{\circ}$C. After that enzyme activity was calculated to these fractions [17] and protein concentration was determined for the most active fraction.

iii-Protein assay:

The protein concentrations in different stages of enzyme purification were estimated according to Bradford [18] using bovine serum albumin as a standard protein.

iv-Electrophoretic analysis:

Small parts of protease samples came from crude culture supernatant and the highest fraction activity of the enzyme from the two steps of purification was freezed and stored to be run in (SDS- PAGE) analysis to confirm the level of purification in each step. All were analyzed against a standard marker protein mixture with known molecular weights according to the protocol [18].

F- Preparation of fungal extract:

The fruiting bodies of *G. lucidum* were collected from around Delta region of Gharbia governorate in August 2014. The extraction method of Kamra and Bhatt [19] was used. The dried fruiting bodies were ground to a fine powder using a domestic blender. For preparing the methanol extract, 1 gram of dried powder was mixed with 50 ml of methanol in 100 ml conical flask. The flasks were plugged tightly with cotton and wrapped with papers. All conical flasks were kept on shaker for 24 h for extraction and allowed to stand for five hours to settle the active compounds. After the completion of extraction, the supernatant was filtered through Whatman no.1 filter paper. Then, the extracted fractions were evaporated to dryness at 40 ºC to obtain residues. For testing the antibacterial activity, the residues were dissolved in Dimethyl Sulfoxide (DMSO) to obtain 10 mg/ml stock solutions. The extracts were stored at 4ºC in air tight containers.

G- Effect of *G. lucidum* extract on ESβLMDRPA:

Antibacterial activity of *G. lucidum* extract was carried out by modified agar well diffusion method [20]. One hundred microliters of inoculum (10^7 cfu/ml) using standard turbidity (corresponding to 0.5 McFarland tube) was applied on the dried surface of prepared nutrient agar plate. For antibacterial screening, *G. lucidum* extract was dissolved in DMSO to a final concentration of (0, 0.5, 1, 1.5, 2, 2.5 mg/ml). Regular wells were made in the inoculated agar plates by a sterile cork borer with 0.8cm diameter. Each well was aseptically filled up with 0.1ml volume of extract. After holding the plates at room temperature for some time to allow diffusion of the extract into the agar, the plates were incubated at 37°C for 24h. After 24 hours, each plate was examined for the zone of inhibition. The tests were performed in triplicates and final values were expressed as mean ± standard deviation.

H- Mode of inhibition of *G. lucidum* extract on *P. aeruginosa* protease enzyme:

In order to detect if *G. lucidum* extract is a protease inhibitor or not; we can culture *P. aeruginosa* on liquid protease dependent medium, as mentioned previously according to Miller [14]. Then, the media was divided into 5 ml fractions in test tubes and sterilized by autoclave. 50 µl of different concentrations of *G. lucidum* extract (0.5, 1, 1.5, 2, 2.5 mg/ml) were separately added to each test tube. Suspension of *P. aeruginosa* (50 µl of inoculum) was inoculated in each tube and incubated at 37°C for 3 days (the optimum time for protease production) and one tube without extract used as control. Then, protease activity was measured quantitatively for each mixture, as described previously [16].Three replica were made for each tested concentration.

Other parameters should be tested to elucidate the mode of inhibition on *P. aeruginosa* by *G. lucidum* extract. Two factors were studied; the first is the concentration of *G. lucidum* extract (as a protease inhibitor), and the second is the concentration of casein (as a substrate for protease), to detect if *G. lucidum* extract is a reversible protease inhibitor or not, arising the mode of its competition with casein. This could be obtained as follows:

Purified protease dissolved in 50 mM Tris-HCl buffer (pH= 7) was used. Different concentrations of *G. lucidum* extract (0.5, 1, 1.5, 2, 2.5 mg/ml) were prepared, and incubated separately with enzyme added to it different concentrations of (substrate) casein (5%, 10%, 15%, 20%, 25%) .Then, the activity of protease enzyme was determined for each substrate and extract concentration quantitively according to Khembavi *et al*. [16] Results were represented graphically by a reciprocal plot between enzyme activity and substrate concentration for each *G. lucidum* extract concentration [21].

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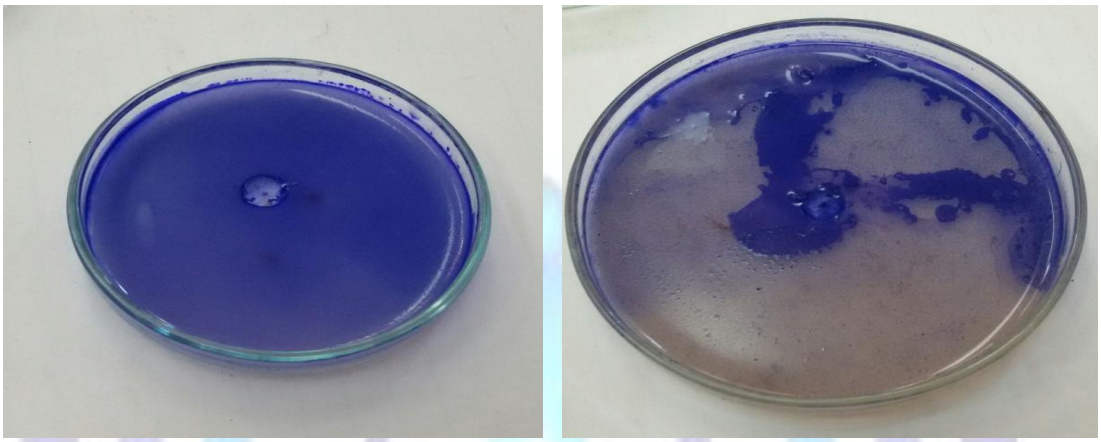
I- Statistical analysis:

In analyzing the data of *P. aeruginosa* protease enzyme production and the effect of *G. lucidum* extract on enzyme activity through analysis of variance (ANOVA), one way tests by SPSS V17 was used, to evaluate the variation among concentrations of *G. lucidum* extract on bacterial growth and its enzyme activity.

RESULTS:

Detection of protease:

Figure (1) reports that *P. aeruginosa* isolate possesses high proteolytic activitiy which appeared as clear areas (no blue colour), indicating the hydrolysis of the bovin serum albumin (BSA) compared to control (blue color of BSA).



Control (non treated with bacteria) **Treated with *P. aeruginosa* on the surface of plate**

Figure (1): Detection of protease enzyme from *P. aeruginosa* isolate

Production of protease:

The production of protease enzyme by *P. aeruginosa* is detected as in table (1). The highest significant rate of protease production (27 unit/ml) is quantitatively reported in the culture filtrate of *P. aeruginosa* on the third day of growth at P<0.001.

Table (1): Time course of protease production by *P. aeruginosa*

Growth age (days)		Enzyme activity (u/ml)
		Protease
1		5±0.5[a]
2		16±1[b]
3		27±1[c]
4		21.7±1.5[d]
5		20±2[d]
6		11.3±1.5[e]
7		6±1[a]
8		2.5±0.5[f]
9		1.6±0.5[f]
10		1±1[f]
ANOVA	F	197.58
	P-value	0.0001

Each value is the mean of three replicates ± SD

Values with the same letter are insignificant at p ≤ 0.05

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Purification of protease:

The extracellular protease produced by *P. aeruginosa* is purified in two steps by 50-70% ammonium sulphate precipitation followed by DEAE-Sephadex A-50 columnn chromatography table (2). The recovered active fraction from 70 % ammonium sulphate of culture broth is adsorbed on the DEAE-Sephadex A-50 matrix. The bound protease is eluted with 10 mM Tris–HCl buffer, pH (7.0). Figure (2) shows that fractions (6-8) are the most active fractions of *P. aeruginosa* protease activity. This is confirmed by appearance of a single protein band at molecular weight 40 kDa during the SDS – PAGE analysis as shown in figure (3). After purification, protease enzyme has indicated specific activity of 84.6 unit/mg from 9 unit/mg with a purification fold of nearly 9.4 times of the enzyme crude as shown in table (3).

Table (2): Protease activity of different precipitates by ammonium sulphate saturations of crude *P. aeruginosa* culture extract:

Amm. sulphate saturation (%)	Protease activity (u/ml)
50	7
60	10
70	39

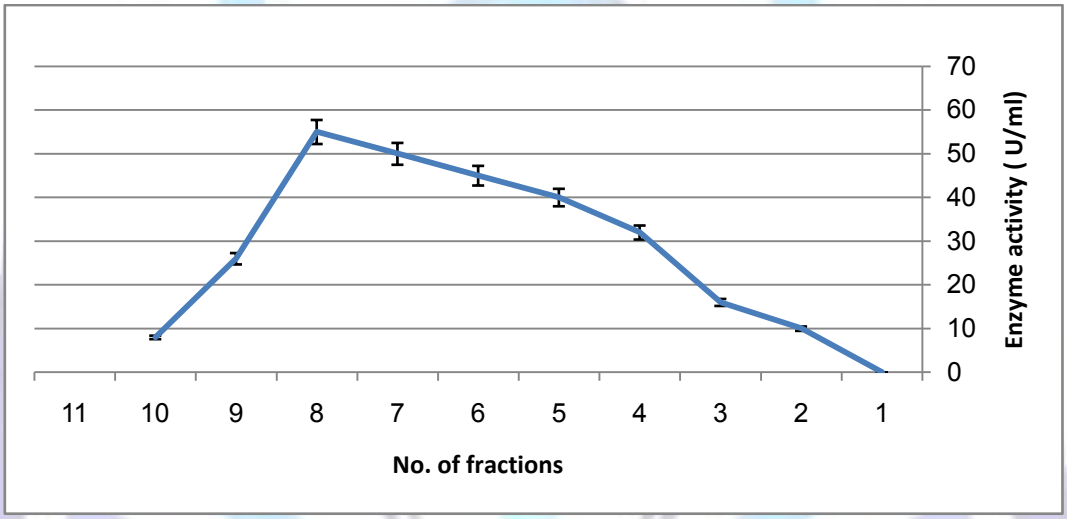

Figure (2): Elution profile of *P. aeruginosa* protease on DEAE-Sephadex A-50.

Table (3): Purification profile of *P. aeruginosa* protease:

Purification parameter	Purification stage		
	Crude culture filtrate	70% amm. sulphate ppt.	DEAE-Sephadex A-50 fraction of highest activity
Total enzyme activity (u)	5400	1950	275
Total protein (mg)	600	50	3.25
Specific enzyme activity (u/mg)	9	39	84.6
Purification fold	1	4.3	9.4
Yield (%)	100	36	5

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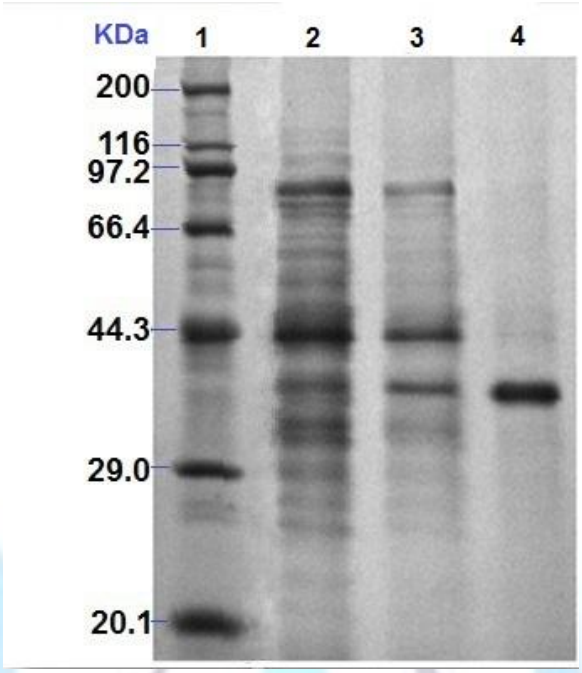

Figure (3): SDS-PAGE for different stages of protease purification.

1= Standard protein marker, 2= Crude culture filtrate, 3=70 % Amm. suphlate precipitate, 4= DEAE-Sephadex A-50of most protease activity.

Effect of *G. lucidum* on bacterial growth and protease activity:

A trial is carried out to find a relationship between high inhibition of *P. aeruginosa* growth by *G. lucidum* extract and protease activitiy as an important offensive force for the bacteria to establish the infection.

Table (4) shows significant differences at P<0.001 on the effect of *G. lucidum* concentrations on the growth of *P. aeruginosa* as concentration of 2 mg/ml is found to be the lowest concentration of *G. lucidum* extract giving the highest inhibitory effect (MIC) on *P. aeruginosa*. By increasing the concentration of *G. lucidum* extract, there is great decrease in protease activity from 27 u/ml to 3 u/ml at MIC concentration (2 mg/ml), as evident in table (4). Also, statistical analysis reveals that the variation in the effect of different concentrations of *G. lucidum* on protease activity is highly significant at P<0.001. Figure (4-A) shows that *G. lucidum* extract is a reversible non-competitive protease inhibitor, as its activity decreases by arising its substrate concentrations which is confirmed with different *G. lucidum* extract concentrations as K_m (Michaelis constant) = 9.7 u/ml. This high K_m shows low affinity between enzyme and substrate. Plotting the slope of the double reciprocal plot of *G. lucidum* extract inverse against the inverse of protease intial activity indicates an inhibition constant (k_{is}) of 0.45 mg/ml due to the effect of *G. lucidum* extract on an enzyme reaction slope, as shown in figure (4-B).

Table (4): Inhibitory effect of *G. lucidum* methanol extract on growth and protease activitiy of *P. aeruginosa*:

Concentration (mg/ml)		Diameter of inhibition zone (mm)	Protease activity (U/ml)
0 5 10 15 20 25		0±0[a] 2±0.25[b] 2.8±0.29[c] 4.6±0.32[d] 7.3±0.47[e] 9.2±0.25[f]	27±2[a] 21±1[b] 18±2[c] 15±1[d] 3±1[e] 0±0[f]
ANOVA	F	402.11	180.65
	P-value	0.0001	0.0001

Each value is the mean of three replicates ± SD

Values with the same letter in the same column are insignificant at p ≤ 0.05

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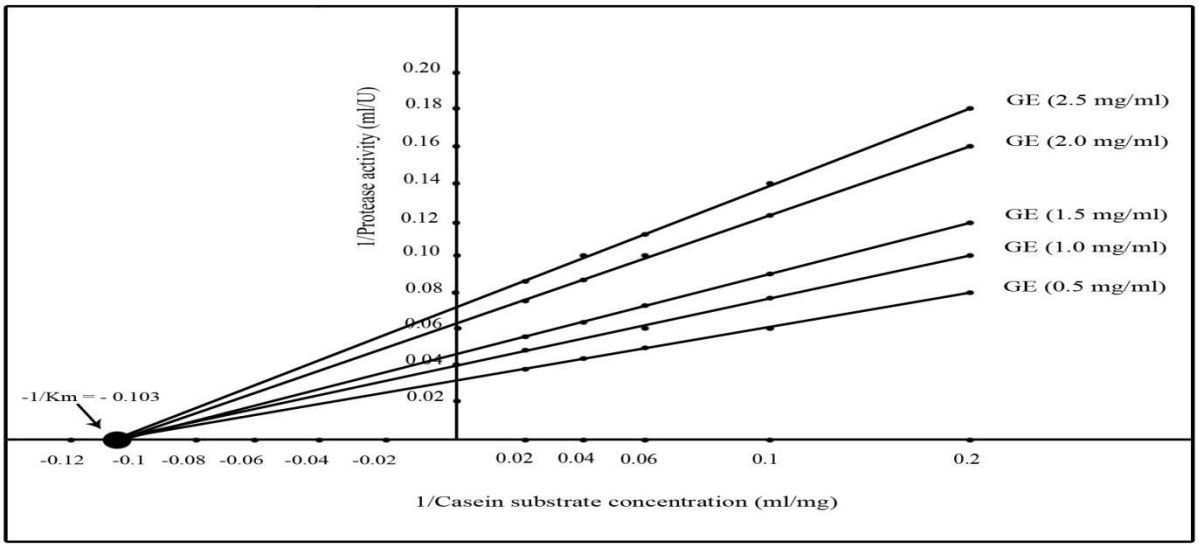
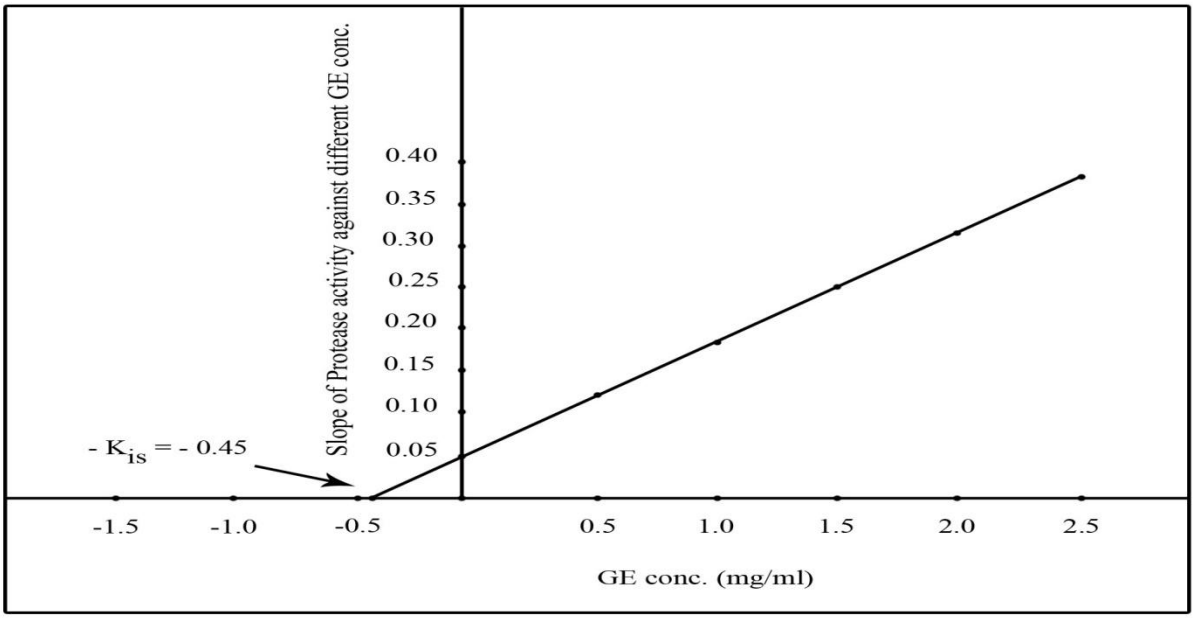


A



B

Figure (4): Inhibition mechanism of *G. Lucidum* extract (GE) against protease activity of *P. aeruginosa*, A: Lineweaver Burk plot, B: Replot of reciprocal protease activity slope against different concentrations of *G. lucidum* extract.

DISCUSSION:

Bacterial growth can be established within human organs by strong offensive forces, such as secretion of extracellular enzymes. *P. aeruginosa* is the first most common pathogen associated with skin burn infection [22]. Virulence of *P. aeruginosa* has been attributed to cell associated factors like alginate, lipopolysaccharide (LPS), flagellum, as well as with exoenzymes or secretory virulence factors like protease [23, 24]. These factors have been shown to play an important role in pathogenesis of *P. aeruginosa* induced infections. So, part of the present study focuses on detecting the ability of isolated bacteria to produce this enzyme, which may facilitate the passage of bacteria into human body causing infection.

In the present work, the major protease yield of *P. aeruginosa* with ammonium sulphate is supported by the results of Kumar *et al*. [25] for Bacillus SNR01 with ammoniom sulphate (65% saturation). However, kumar *et al*. [26] report that protease in the culture supernatant of *Bacillus thuringensis* was precipitated with 35% ammonium sulphate saturation.

SDS – PAGE analytical studies of protease reveal that it possesses a single protein band with molecular weight of 40 kDa. This agrees with *P. aeruginosa* by Gupta *et al*. [27] . On the other hand, Rahman *et al*.[28] represent protease of *P. aeruginosa* as a single band with molecular weight of 29 kDa upon SDS – PAGE analysis.

Inhibition of protease by *G. lucidum* extract, as an important offensive force against *P. aeruginosa* leads to decreasing the ability of the bacteria to cause infection. In accordance with our results, Prasad *et al*.[29] detect a potential protease inhibitory activity of *Achaea janata*. But, they report the moderate inhibition of protease enzyme by *Vigna mungo.*

In a trial to explain the ability of *G. lucidum* extract to manage infection, it was observed that *G. lucidum* extract has strong antiprotease activity. Our study determines that *G. lucidum* extract kinetics properties act as irreversible competitive inhibitor for protease enzyme, in accordance with Arulpandi *et al*. [30] who report reversible non-competitive inhibition of protease by using *Cassia fistula* leaf extract. In contrast to our results, El Zawawy [31] represent the reversible-competitive inhibition of keratinase by *Anethum graveolens* seed extract.

Conflict of interest:

The authors declare that they have no conflict of interest .

REFERENCES:

1- Ullah A., Durrani R., Ullah I. and Rafiq M. (2012) Antibiotic resistance profile of clinical Gram-negative bacteria. J.Biol. Food Sci. Res.1(2): 23-25.

2-Morales-Espinosa R., Soberon-Chavez G., Delgado-Sapien G. and Sandner-Miranda L. (2012) Genetic andphenotypic characterization of a Pseudomonas aeruginosa population with high frequency of genomic islands. PloS ONE. 7(5): e37459.

3-Timani R J. (2012) Molecular typing and biofilm characterization of *Pseudomonas aeruginosa* isolated from clinical samples in Lebanon.

4- Leidal KG, Munson KL, Johnson MC, Denning GM. (2003) Metalloproteases from *Pseudomonas aeruginosa* degrade human RANTES, MCP-1, and ENA-78. J Interferon Cytokine Res. 23:307-318.

5- Shibuya Y, Yamamoto T, Morimoto T, Nishino N, Kambara T, Okabe H. (1991) *Pseudomonas aeruginosa* alkaline proteinase mightshare a biological function with plasmin. Biochim Biophys Acta.1077:316-324**.**

6- Rumbaugh KP, Griswold JA and Hamood AN. (2000) The role of quorum sensing in the in vivo virulence of Pseudomonas aeruginosa. Microbes Infect. 2:1721-1731.

7-Kipnis E., Sawa T. and Wiener-Kronish, J. (2006) Targeting mechanisms of *Pseudomonas aeruginosa* pathogenesis. Med. Mal. Infect. 36: 78-91.

8-Bielecki P., Glik J., Kawecki M. and Martins dos Santos VA. (2008) Towards understanding Pseudomonas aeruginosa burn wound infections by profiling gene expression. Biotechnol. Lett. 30:777-790.

9- Klaus A, Miomir N (2007) Influence of the extracts isolated from *Ganoderma lucidum* mushroom on some microorganisms. *Proceedings of National Science, Matica Srpska Novi Sad*, 113: 219-226**.**

10- Ha TB, Gerhauser C, Zhang WD, Ho-Chong-Line N, Fouraste I (2000) New lanostanoids from *Ganoderma lucidum* that induce NAD(P)H: quinone oxidoreductase in cultured hepalcic7 murine hepatoma cells. Planta Medica*,* 66(7): 681-684.

11- Chang ST, Mshigeni KE (2001) Muschroom and Human health: their growing significance as petent dietary supplements. University of Namibia, Windhoek, Namibia. pp:79.

12- Khalil M., Sonbol F. Badr A. Ali S. (2015) Comparative study of virulence factors among ESβLs producers and non producers *Pseudomonas aeruginosa* clinical isolates. Turk. J. Med. Sci. 45(1) 60-69.

13- Vermelho A., Meirelles M. and Lopes A. (1996) Detection of Extracellular Proteases from Microorganisms on Agar Plates. Mem Inst Oswaldo Cruz, Rio de Janeiro 2 (6): 755-760.

14- Miller JH. (1972) Experiments in Moleculer Genetics. Cold Spring HarborLaboratory, Cold Spring Harbor, NY 1972; pp. 431-5.

15-Ferrasson E. Quillien L. Gueguen J. (1997) Proteinase inhibitors from pea seeds, purification and characterization. J. Agric. Food Chem. 45 (1): 127–131.

16- Khembhavi A. kulharni A., and Pant A. (1993) Salt tolerant thermostable alkaline protease from Bacillus subtilis NCIM NO. 64. Applied Biochemistry and Biotechnology 38, 83-92.

17- Jellouli K., Bayoudh A., Manni L., Agrebi R. and Nasri M. (2008) Purification, biochemical and molecular characterization of a metalloprotease from *P. aeruginosa* MN7 grown on shrimp wastes. Appl. Microbiol. Biotechnol. 79, 989-999.

18- Bradford M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal.Biochem., 72: 248-254.

19- Kamr A., and Bhatt AB. (2012) Evaluation of antimicrobial and antioxidant activity of *Ganoderma lucidum* extracts against human pathogenic bacteria. International Journal of Pharmacy and Pharmaceutical Sciences.4(2): 359- 362.

20- Quereshi S, Pandey AK, Sandhu SS. (2010) Evaluation of antibacterial activity of different Ganoderma lucidum extracts. People Journal of Scientific Research 3(1): 9-13.

21- Lineweaver H. and Burk D. (1934) The determination of enzyme dissociation constants. J. Ameri. Chemi. Soci. 56: 665-666.

22- Jarvis WR, Martone WJ. (1992) Predominant pathogens in hospital infections. J Antimicrob Chemother 29:19-24.

23-Matheson NR, Potempa J, Travis J. (2006) Interaction of a novel form of *Pseudomonas aeruginosa* alkaline protease (aeruginolysin) with interleukin-6 and interleukin-8. Biol Chem 387:911-5.

24- Veesenmeyer JL, Hauser AR, Lisboa T, Rello J. (2009) *Pseudomonas aeruginosa* virulence and therapy: evolving translational strategies. Crit Care Med 37:1777-86.

25- Kumar C.G.; Tiwari M.P. and Jany K. D. Proc.(1999) Biochem. 34: 441-449.

26- Kumar A.; Sachdev A.; Balasubramanyam S.; Saxena A. and Lata L. (2002) Optimization of conditions for production of neutral and alkaline protease from species of Bacillus thuringensis. Ind. J. Microbiol., 42: 233-236.

27- Gupta A, Roy I, Khare SK, Gupta MN.(2005) Purification and characterization of a solvent stable protease from *Pseudomonas aeruginosa* PseA. J Chromatogr 1069: 155-61.

28- Rahman R, Geok LP, Basri M, Salleh AB. (2006) An organic solvent-stable alkaline protease from Pseudomonas aeruginosa strain K: Enzyme purification and characterization. Enzyme Microb Technol 39:1484-91.

29- Prasad E.R., Dutta-Gupta A., Padmasree K. (2010) Insecticidal potential of Bowman-Birk proteinase inhibitors from red gram *(Cajanus cajan*) and black gram (*Vigna mungo*) against lepidopteran insect pests. Pestic. Biochem. Physiol. 98 (1): 80–88.

30- Arulpandi S and Sangeetha R.(2012) Antibacterial Activity of Fistulin: A Protease Inhibitor Purified from the Leaves of Cassia fistula. ISRN Pharmaceutics 35: 88-95.

31- El Zawawy N. (2010) Pathophysiological studies among different yeast isolates causing tinea versicolor. M.Sc. Thesis, Bot. Dept., Faculty of Science, Tanta University, Egypt.